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Monoclonal Antibodies Recognizing Surface Residues of the β Subunit of *Escherichia coli* F_1 ATPase: Functional Importance of the Epitope Residues[1]

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Two monoclonal antibodies, β 208 and β 210, against the β subunit of the F_1 ATPase from *Escherichia coli* reacted with an intact β subunit and also a peptide corresponding to a portion of β between residues 1 and 145. Mutations at Ala-1, Val-15, Glu-16, Phe-17, Leu-29, Gly-65, or Leu-66, and His-110 or Arg-111 for β 210 and β 208, respectively, caused decreased antibody binding to β, suggesting that these residues form the epitopes and are thought to lie close together on the surface of the β subunit. The topological locations of the corresponding residues in the atomic structure of the bovine β subunit agree well with these expectations, except for Ala-1 and Leu-29. β 210 binds to two β strands including the epitope residues that are 50 residues apart, indicating that this antibody recognizes the tertiary structure of the N-terminal end region. Mutations in the epitope residues of β 210 do not affect the F_1 ATPase activity, suggesting that surfaces of the two β strands in the amino-terminal end region are not functionally essential. To analyze the functional importance around His-110 recognized by β 208 we introduced site specific mutations at residues His-110 and Ile-109. Ile-109 to Ala or Arg, and His-110 to Ala or Asp caused defective assembly of F_1. However, the His-110 to Arg mutation had no effect on molecular assembly, suggesting that Ile-109 and His-110, especially the positive charge of His-110 are essential for the assembly of F_1. The His-110 to Arg mutation caused a large decrease in F_1-ATPase activity, suggesting that a subtle change in the topological arrangement of the positive charge of His-110 located on the surface of β plays an important role in the catalytic mechanism of the F_1-ATPase.

Key words: epitope mapping, F_1-ATPase, monoclonal antibodies, site directed mutagenesis.

The proton translocating ATPase (F_1F_0) plays a key role in energy transduction in living cells (*1–7*). The structure of this enzyme is similar among various organisms and exists on energy transducing membranes such as mitochondria, bacteria, and chloroplast membranes. The enzyme from *Escherichia coli* is composed of a membrane integral portion (F_0) with three different subunits, a, b, and c, and a membrane peripheral portion (F_1) with five different subunits, α, β, γ, δ, and ε. The F_1 portion has the ATPase activity while F_0 has an ion channel activity. The β subunit has a catalytic site with sequences that are highly homologous among various organisms. The coupling mechanisms of the proton transfer through F_0 and the catalysis of ATP synthesis or hydrolysis are important issues to be solved at present.

The ATPase activity has been reconstituted from purified *E. coli* α, β, and γ subunits (*8*, *9*). Based on the atomic structure of bovine α and β, and a portion of γ, the γ subunit has been shown to contain long α helical structures at the N- and C-terminal regions (*10*). These α helical segments run through the central cavity surrounded by three pairs of α and β. The γ and ε subunits have been shown to rotate within the cavity as a shaft for thermophilic *Bacillus* PS3 ATPase (*11*, *12*) and *E. coli* (*13*). Moreover, the membrane integral part composed of subunit c has been shown to rotate (*14*).

To understand the rotation mechanism in the ATPase, information about dynamic structural changes in the β subunit that can not be obtained by a static structure based on an analysis of atomic structure alone is required. For the expected dynamic structural changes, amino acid residues exposed on the molecular surface of β may play important roles in terms of inter-subunit or intra-subunit interactions of the domains in the subunit. From this point of view, we reported a new, potentially effective approach combined with monoclonal antibody and analysis of mutated residues for the epitope (*15–17*). This approach provides a tool for detecting residues located on the surfaces of subunit mole-

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cules and conformational changes in the surface residues, leading to functional alterations of the F_1-ATPase. Here we obtained new monoclonal antibodies against β that recognize the amino-terminal end region and residues around 110 located close to a connecting region between the central catalytic domain and the amino terminal β barrel region in β (*10*). As a result, the positive charge of His-110, which is apart from the catalytic domain, has been revealed to be an important site for the catalytic mechanisms of the F_1-ATPase.

MATERIALS AND METHODS

E. coli Strains and Culture Conditions—*Escherichia coli* strains KM230 (wild type, *thy*, *thi*, *asn*) (*18*) and DK8 (*bgl*R, *thi*-1, *rel*-1, Δ*unc*B-C, *ilv*:Tn10) (*19*), or JP17 (Δ*unc*D, *arg*H, *pyl*E, *ent*A, *rec*A) (*20*) were used. *E. coli* cells were grown in minimal medium (Tanaka medium) supplemented with succinate, glycerol or glucose as the sole carbon source or rich medium (L broth) at 37°C with vigorous shaking (*18*). Transformants with various plasmids were selected on rich-medium agar (L-broth) (*18*) containing ampicillin (50 μg/ml) or appropriate antibiotics.

Preparation of Hybridoma, Monoclonal Antibodies against the β Subunit, and Immunological Assays—Thirty-five micrograms of the purified β subunit protein was injected into BALB/c mice, which were further immunized over a period of 2 months with three booster injections at three-week intervals. Construction of hybridomas producing the antibodies was performed as described previously (*21*). Monoclonal hybrid cells of the myeloma cell line NS-1 and immunized spleen cells were selected as HAT-resistant clones. Hybridoma cells producing the antibodies were propagated as ascites. The antibodies prepared in this study were partially purified by ammonium sulfate precipitation as described previously (*21*). The immunological reactivities were assayed by enzyme-linked immunosorbent assays (ELISA) with 0.08 μg of the purified β subunit per well. Total *E. coli* cell proteins (2.5 μg per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Millipore GVHP membranes (*21*). The β subunit was visualized by incubation with the antibodies and a subsequent ABC procedure using a Vectastain kit (*21*).

Construction of an Expression Plasmid of the β Subunit with Mutations and Immuno-Screening of Mutants Incapable of Binding the Antibodies—For the construction of an expression vector of the β subunit with mutations, pYS21 (*22*) was used. Random mutations were introduced into the region between residues 1 and 145 of β by two step PCR (*23*) as described previously (*24*). For the first PCR to induce mutations, the entire gene of the β subunit was amplified with primers, GF 3 (5′-GCGTGTCGTTCTTCAACTCC-3′) at the 5′ end and β 2 (5′-TAACACCGGCTTGAAAAGC-3′) at the 3′ end, and template plasmid, pKM01 (*15*). For the first 5 cycles, the concentration of one substrate in the PCR reaction was decreased to 0.4 μM instead of 200 μM to induce mutations. PCR conditions were basically the same as described previously (*24*): each cycle: 1 min at 94°C for denature, 2 min at 45°C for annealing, and then 3 min at 72°C for elongation with Tth DNA polymerase (*24*). Following the first 5 cycles, 25 cycles were performed during which the substrate was added to the 200 μM level. Following the first PCR, the second PCR was performed with internal primers of the β subunit, GF3 and BR6 (5′-CGTCAGGAATTTCTCAGCC-3′), and *Pfu* DNA polymerase. The amplified DNA was digested with restriction endonucleases *Hin*dIII and *Nco*I, and the resulting fragment was replaced by the corresponding region in the expression plasmid pYS 21. The resulting pYS 21 derivatives were introduced into *E. coli* DK8. Colonies of these cells grown on L broth agar were transferred to nylon membranes and the immuno-reactivities of cell materials in each colony were analyzed as described previously (*21*). From the colonies that did not show reactivities, plasmids were extracted. Mutations within the coding region of the β subunit in the plasmids were determined by sequencing DNA following the dideoxy chain termination method (*25*).

Characterization of the β Subunit Mutants—To measure the F_1-ATPase activity, JP 17 cells transformed with the expression plasmid of β with a mutation were grown in 400 ml of Tanaka medium containing glycerol as the carbon source and harvested in the middle-logarithmic phase of growth. The membranes were prepared and the ATPase activity was measured as described previously (*26*). For detection of the molecular assembly of F_1, membrane proteins (2.0 μg) from JP 17 carrying pYS 21 or pYS 21 with various mutations were subjected to polyacrylamide gel electrophoresis (12.5% polyacrylamide) with SDS, and the separated proteins were blotted onto nylon membranes (Millipore GVHP). The α and β subunits were detected with monoclonal antibodies α108 and β31 (*15*, *16*), respectively. The γ subunit was detected by polyclonal antibodies raised against the subunit (*27*).

Site Directed Mutagenesis—Two step PCR was used to introduce site directed mutations at residues 109 and 110. For the first PCR, two regions in the β subunit gene were amplified. One segment is between residues 109 or 110, and residue 355 (nucleotide position 1066 from the 5′ end of the β subunit gene). The reverse primer at position 1066 (BR 2) (*24*) and the forward primer containing a substituted nucleotide at residue 109 or 110 were used. The mutated primers were as follows: Ile-109 to Ala, 5′ GTTGGGCGGCTCACCGCGCAGCGCCTTCCTA 3′; Ile-109 to Arg, 5′ GTTGGGCGAGACACCGCGCAGCGCCTTCCTA 3′; His-110 to Ala, 5′ GGGCGATTGCACGCGCAGCGCCTTCCTA 3′; His-110 to Asp, 5′ GGGCGATTGATCGCGCAGCGCCTTCCTA 3′. The bases altered to achieve the replacements are underlined. An additional underline at G indicates that this G was introduced to create a *Hae*II site, causing a silent mutation. This site was used to confirm the introduction of a mutation in the amplified DNA. Another DNA segment between residues 1 and 220 was amplified with primers β1 and BR 6 (*24*). The resulting two DNA segments amplified were used for the second PCR as the primers. Products of the second PCR between residues 1 and 355 were digested with *Sal*I and *Sac*I. The *Sal*I and *Sac*I fragment was integrated into the corresponding region in pYS 21. The introduction of mutations in the recombinant pYS 21 plasmids was confirmed by digestion with *Hae*II and further DNA sequencing.

Other Procedures—The F_1-ATPase was purified from *E. coli* KM 230 as described previously (*18*). Preparation of plasmid DNAs and digestion with restriction endonucleases, ligation of DNA fragments with T4 ligase and other procedures for the manipulation of the DNA were per-

formed according to published procedures (*28*). Proteins were measured as described previously (*29*).

Materials—Restriction enzymes, *Tth* and *Pfu* DNA polymerase, and T4 ligase were purchased from Toyobo (Osaka), and New England Biolabs (Beverly). α-^{35}S-dCTP was purchased from Dupont/NEN Research Products. The oligonucleotides used in this study were synthesized on a DNA synthesizer (Applied Biosystems model 380A). Other materials were of the highest grade commercially available.

RESULTS

Epitope Mapping—We prepared eight independent monoclonal antibodies by a standard method of hybridoma construction (*21*) including enzyme-linked immunosorbent assay to detect antibody-production. The antibodies were purified from the ascites of a mouse inoculated with the hybridoma. An epitope area on the β subunit was localized based on reactivities of various portions of β peptides with the antibodies (Fig. 1). As shown in Fig. 1, all of the antibodies recognized the N-terminal half region except for one (β 205), which recognized the C-terminal end region. Since the primary structures of the β subunit are highly conserved between *E. coli* and mammals (*5*), and the functional importance of the central portion of the β subunit between residues 202 and 365 is known (*1, 2, 5, 6, 10*), hybridomas producing antibodies against this region may not be viable.

Identification of Epitope Residues for β 208 and β 210 Monoclonal Antibodies—We have further analyzed antibodies β 208 and β 210. These antibodies did not inhibit the ATPase activity of the purified F_1-ATPase. To show whether the epitope residues for these antibodies reside on the surface of the F_1-ATPase complex, we performed competition assays with F_1 in the binding of the antibodies and the purified β. We showed previously that this type of experiment in the cases of monoclonal antibodies β 12 and β 31 work, and concluded that the epitope residues for these antibodies are within the F_1 complex (*21*). Here we performed the same type of experiments for β 208 and β 210. The binding of the both antibodies to β was inhibited by the purified F_1-ATPase to some extent, but the results were not necessarily reproducible. The reason for this might be that the locations of the epitope residues in the F_1 complex are partly exposed on the surface of the F_1 complex. Next we estimated the epitope residues in the β subunit following the same protocol to identify the epitope residues as described previously (*15*). An outline of the procedures is as follows: random mutations were introduced into peptides 1–104 and 47–145, for β 210 and β 208, respectively. *E. coli* colonies carrying mutated peptides were surveyed for their reactivity with the two antibodies. In expression plasmids showing reduced immuno-reactivity, the altered nucleotide sequence was identified by sequencing the DNA.

Twelve out of 4,520 independent clones and six out of 3,294 independent clones that carried β expression plasmids showed no binding activities to β 210 and β 208, respectively (Table I). Typical results of the immuno-reactivity of monoclonal antibody β 210 are shown in Fig. 2. Monoclonal antibody β 209, which recognized residues between 146 and 201, bound to all of the mutated β subunits, confirming the presence of β in the mutant cells. These results indicate that mutations in residues between 1 and 104 cause decreased binding activities of the peptide to β 210. Similarly, for monoclonal antibody β 208, reduced binding activities were observed for 6 peptides carrying mutations between residues 47 to 145. Again the presence of mutated β was confirmed by monoclonal antibody β 209 (data not shown).

For β 210, twelve independent clones with mutations were isolated. Altered nucleotide sequences were identified for the mutant peptides by DNA sequencing of the expression plasmids. As a result, nine mutations were identified

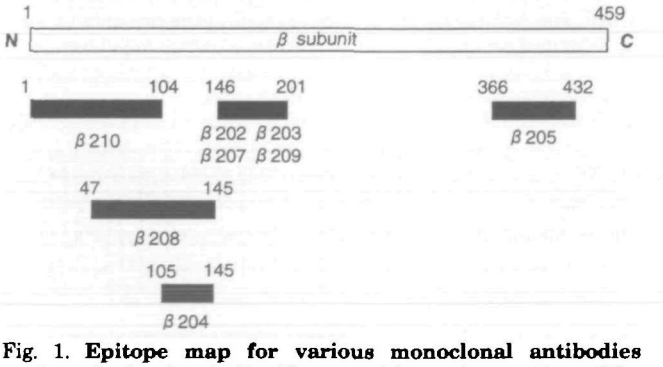

Fig. 1. **Epitope map for various monoclonal antibodies against the β subunit.** Peptides comprising various portions of the β subunit shown by black bars were overexpressed in *E. coli* (BL 21) with an expression plasmid (pET 3d or 3xa) (*21*) carrying those portions. Extracts of these cells were blotted onto nylon membrane filters after SDS-polyacrylamide gel electrophoresis, and incubated with an antibody. Based on the results (not shown), the estimated epitope sites for each monoclonal antibody are shown. Since the first Met is not present in the purified β subunit, residue 1 is Ala.

TABLE I. **Isolation of mutants by region-specified PCR mutagenesis in the *unc*D gene.**

mAb	Total no. of[a] colonies	Candidate[b] colonies	Candidates[c] checked	Mutants[d]
210	4,520	62	19	12
208	3,294	48	19	6

[a]Ampicillin-resistant transformants appeared after transfection of expression plasmids with a mutation to JP17. [b]Colonies that showed weaker binding to mAb in immunochemical screening. [c]Clones selected randomly from the candidate colonies for further analysis by Western blotting and screening. [d]Clones in which decreased binding of β to the antibodies was observed.

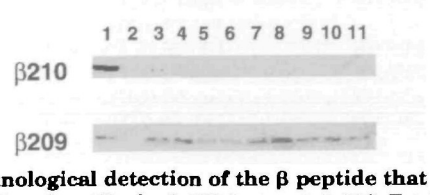

Fig. 2. **Immunological detection of the β peptide that reacted with monoclonal antibody β 209 but not β 210.** Total cell extracts of *E. coli* (10 μg) carrying expression plasmids of β with a mutation were subjected to SDS–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were blotted onto nylon membrane filters and incubated with β 209 or β 210. The reacted materials were visualized using an ABC Vecta stain kit as described previously (*21*). Lane 1, wild-type (JP 17 transformed by pYS 21 with the wild-type β); Lane 2, control JP 17 without β; Lane 3, plasmid 210-2; Lane 4, plasmid 210-5; Lane 5, plasmid 210-6; Lane 6, plasmid 210-14; Lane 7, plasmid 210-27; Lane 8, plasmid 210-104; Lane 9, plasmid 210-105; Lane 10, plasmid 210-106; Lane 11, plasmid 210-107. Plasmid numbers are the same as in Table II.

at residues 1, 15, 16, 17, 29, 65, and 66 (Table II) (*15*), including mutations at the same residues (residues 17 and 66) in the region between residues 1 and 104. These results suggest that these seven residues are involved in the epitope of β 210. Besides these nine single mutations, double mutations were found for three expression plasmids, 210-1, 210-101, and 210-103. Since the nine candidate sites for epitopes are separated in the primary structure of β, this antibody might recognize the tertiary structure of the amino-terminal end portion of β.

For β 208, four independent single mutations and two double mutations were identified (Table III). The results suggest that the β 208 epitope exits around Trp-107, His-110, and Arg-111. Since β 208 and β 210 bound to an intact β subunit in the enzyme-linked immunosorbent assay, the epitope candidate residues identified for these antibodies were thought to be on the surface of the β subunit.

To confirm that the epitope residues are specific for β 210 or β 208, we tested the reactivities of the mutated β peptides with monoclonal antibodies, β 12 and β 31. We have reported that β 12 and β 31 recognize residues 26, 40, 52, 71, and 74 and 40, 41, 43, 44, 46, and 56, respectively (*15*). In fact, β with mutations at the residues shown in Tables II and III did react with these antibodies except for Phe-17 to Ser mutation for β 12 (data not shown), confirming that the candidate epitope residues estimated here are specific for β 210 and β 208. A Phe-17 to Ser mutation might affect indirectly the epitope site for β 12. Thus antibodies β 210 and β 208 were concluded to be capable of recognizing limited areas in the amino-terminal end region of the β subunit.

Effects of the Mutations at Antibody-Binding Sites on F_1F_0 ATPase—To analyze the function of the residues recognized by β 208 or β 210, we expressed the β subunits defective in binding to the monoclonal antibodies in *E. coli* JP17, a deletion mutant of β. None of the mutants for β 210 showed any defects in growth on minimal medium agar containing succinate as the sole carbon source, on which plates mutants with a defect in F_1F_0 ATPase could not grow. However, among β mutants defective in binding to β 208, a mutant with Arg-111 substituted by Pro exhibited defective cell growth on minimal agar plates supplemented with succinate as the sole carbon source (Table IV). Arg-111 substituted by Ser and His-110 substituted by Arg did not affect the growth on agar plates containing succinate. The F_1-ATPase activity in the membranes from the mutants were measured (Table IV). As expected replacement of Arg-111 by Ser did not cause defects in the activity, while the replacement of Arg-111 by Pro caused a severe decrease of the activity. Although growth on minimal medium supplemented by succinate was observed for the His-110 to Arg substitution, extensive loss of the ATPase activity was observed, suggesting that catalytic mechanism is impaired by this mutation. Residual F_1F_0 activity might support cell growth on a minimal plate supplemented with succinate (Table IV).

Mutations Specified at Residues 109, 110, and 111—As shown in Fig. 3, residues 109 and 110 are conserved in the β subunit from various species, but not residue 111, which is consistent with the notion that Arg-111 is not essential for the F_1-ATPase. Then we further analyzed the effects of mutations at the conserved His-110 and Ile-109 by site

TABLE II. **Mutations affecting the binding of monoclonal antibody β 210 in β peptide.**

Plasmid	Mutation
210-1	Gly-65 (GGT) → Asp (GAT)
	Pro-78 (CCG) → Leu (CTG)
210-2	Val 15 (GTC) → Leu (CTC)
210-5	Ala 1 (GCT) → Val (GTT)
210-6	Phe 17 (TTC) → Ser (TCC)
210-14	Phe 17 (TTC) → Leu (CTC)
210-27	Leu 66 (CTG) → Pro (CCG)
210-101	Arg-63 (CGT) → His (CAT)
	Gly-65 (GGT) → Ser (AGT)
210-103	Ala-1 (GCT) → Asp (GAT)
	Val-39 (GTG) → Met (ATG)
210-104	Glu 16 (GAA) → Lys (AAA)
210-105	Leu 29 (CTT) → Pro (CCT)
210-106	Gly 65 (GCT) → Asp (GAT)
210-107	Leu 66 (CTG) → Pro (CCG)

TABLE III. **Mutations affecting the binding of monoclonal antibody β 208 in β peptide.**

Plasmid	Mutation
208-5	Arg 111 (CGC) → Pro (CCC)
208-104	His 110 (CAC) → Arg (CGC)
208-111	His 110 (CAC) → Arg (CGC)
208-113	Arg 111 (CGC) → Cys (TGC)
	Gly 143 (GGT) → Ser (AGT)
208-409	Arg 111 (CGC) → Ser (AGC)
208-419	Trp 107 (TGG) → Cys (TGT)
	Glu 124 (GAA) → Gly (GGA)

TABLE IV. **Succinate-dependent growth of mutants and ATPase activities on the mutant membranes.**

Plasmid	Growth	ATPase activity (relative)	
pYS21 (wild)	+	5.4	(100)
pBR322 (non ATPase)	–	0.076	(1.5)
pYS21-βHis110 Arg	+	0.94	(18)
pYS21-βArg111 Pro	–	0.28	(5.2)
pYS21-βArg111 Ser	+	6.8	(127)

Plasmids carrying each mutation were introduced into *E. coli* JP17, and transformants showing Ampicillin-resistance were replicated on Tanaka-medium agar supplemented with succinate or glucose as the sole carbon source. Growth was judged in the presence of succinate after 3 days of incubation. Membranes vesicles were prepared as described under "MATERIALS AND METHODS." The ATPase activities were assayed in the presence of Mg^{2+} as described previously. Relative ATPase activities are shown.

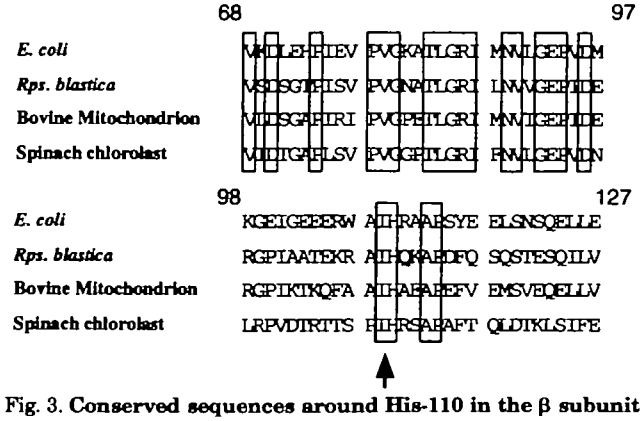

Fig. 3. **Conserved sequences around His-110 in the β subunit of various species.** Conserved sequences are shown by boxes.

TABLE V. **The effect of mutations at residues 109 and 110 in the β subunits upon ATPase activities on the mutant membranes.**

Plasmid	ATPase activity (relative)
pYS21 (wild)	100
pBR322 (non ATPase)	3.6
pYS21-βIle109 Ala	4.5
pYS21-βIle109 Arg	6.1
pYS21-βHis110 Ala	22
pYS21-βHis110 Asp	2.3

Mutations were introduced by PCR-mediated mutagenesis as described under "MATERIALS AND METHODS." ATPase activities on the mutant membranes were assayed in the presence of Mg^{2+} as described previously (*18*). Relative ATPase activities are shown.

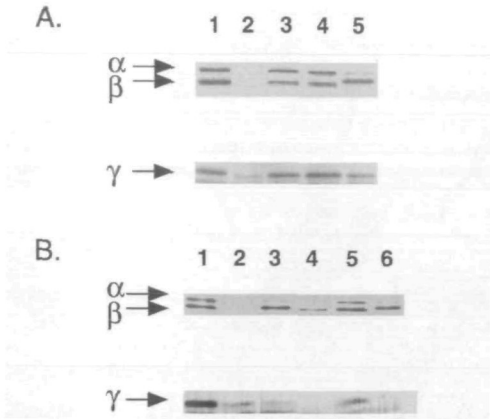

Fig. 4. **Molecular assembly of the α, β, and γ subunits on mutant membranes.** Various expression plasmids of pYS 21 with mutations were introduced into *E. coli* JP 17. Membranes were prepared from the transformants as described in "MATERIALS AND METHODS." For detection of the α, β, and γ subunits, 2.5 μg membrane proteins prepared from cells carrying the wild-type (JP 17 with pYS 21) or mutant plasmid were blotted onto GVHP filters after polyacrylamide gel electrophoresis and reacted with monoclonal antibodies against α or β, or polyclonal antiserum against γ (*15*, *16*, *21*). The reacted materials were visualized by an ABC Vectastain kit as described previously (*21*). A. Lane 1, JP 17 with pYS 21; Lane 2, control JP 17 without β; Lane 3, His-110 to Arg; Lane 4, Arg-111 to Ser; Lane 5, Arg-111 to Pro. B. Lane 1, JP 17 with pYS 21; Lane 2, control JP 17 without β; Lane 3, Ile-109 to Ala; Lane 4, Ile-109 to Arg; Lane 5, His-110 to Ala; Lane 6, His-110 to Asp.

directed mutagenesis. All of the replacements of Ile-109 or His-110 (Ile-109 to Ala or Arg and His-110 to Ala or Asp) resulted in no growth on minimal succinate agar (not shown). F_1-ATPase activities in the mutant membranes were affected by the replacement of Ile-109 with Ala or Arg, and the replacement of His-110 with Ala or Asp also caused an extensive decrease in the ATPase activity (Table V). Next, we analyzed the effects of amino acid replacements at residues Ile-109, His-110, and also Arg-111 on the molecular assembly of the F_1 ATPase by testing for the presence of α, β, and γ in the membranes (Fig. 4, A and B). All mutations accompanied by a loss of F_1-ATPase activity except for the replacement of His-110 with Arg (Fig. 4A, Lane 3), showed significant decreases in α and γ. For the Arg-111 to Pro mutation, decreased but significant amounts of γ were observed (Fig. 4A, Lane 5). These results indicate that the mutations tested cause defects in F_1 assembly except for the His-110 to Arg mutation. The β subunit was observed in the membranes for most mutants as for the wild type. This had been also found for other mutations of β as reported previously (*15*). When the wild type β was expressed in *E. coli*, DK 8, a deletion mutant of the whole ATPase operon, β was found in the membrane fraction, suggesting that β alone or structurally altered β has a tendency to bind nonspecifically to the lipid bilayer (H. Kanazawa, unpublished observation). These results are consistent with the loss of ATPase activity for these mutations. Although extensive loss of the F_1-ATPase activity was found for the replacement of His-110 by Arg, the molecular assembly of F_1 was in tact.

Replacements of Ile-109 by Ala or Arg caused defects in the molecular assembly of the F_1-ATPase (Fig. 4B, Lanes 3 and 4). However, these mutations did not cause any decrease in antibody binding of β for β 208 (not shown), suggesting that Ile-109 is not involved with the epitope residues for β 208.

DISCUSSION

We reported previously that an approach with monoclonal antibodies against the α or β subunits of F_1 ATPase and epitope mapping of the antibodies is useful to detect the surface residues of these subunits (*15–17*). Further, analysis of the effects of mutations at their epitope residues provides a new approach to estimate functions of the molecular surfaces of α and β. Here we isolated eight new monoclonal antibodies against the β subunit and the epitope residues for two of them (monoclonal antibodies β 210 and β 208) were estimated.

Since the antibodies are reactive to an intact β subunit as shown by enzyme-linked immunosorbent assay, the locations of the estimated epitope residues, Ala-1, Val-15, Glu-16, Phe-17, Leu-29, Gly-65, and Leu-66 for β-210, and His-110 and Arg-111 for β 208, were estimated on the molecular surface of β. In fact, the residues in bovine F_1 β corresponding to these residues were found on the surface of the bovine β molecule (*10*) (Fig. 5). Since the primary structures of β in *E. coli* and bovine are highly homologous, these results indicate that β 210 recognizes a tertiary structure of two β strands containing residues 15–17 or residues 65 and 66 in the N-terminal end region (Fig. 5). Amino acid substitutions at residues 1 and 29 also caused decreased binding of β 210. However, they are not on the two β strands, suggesting that residues 1 and 29 might cause indirect effects on antibody binding. For both β 210 and β 208, decreased binding of β to the antibodies was observed for several double mutations (Tables II and III). At present, it is difficult to conclude whether these residues (Val-39, Arg-63, and Pro-78 for β 210, and Trp-107, Glu-124, and Gly-143 for β 208) are involved in the epitope residues of β 210 or β 208. The significance of these residues as epitope residues will be clarified by introducing single mutations at these residues. β 210 and β 208 are useful tools to detect conformational changes and functions of the surface domains of the β subunit containing these residues.

In bovine β, about 80 residues in the amino-terminal end region comprise 6 β-sheets and form a β barrel structure (*10*). About 110 residues following this β barrel comprise α helices and β sheets and form a nucleotide binding site as the catalytic center. In steady state catalysis, the γ subunit rotates in the center of the catalytic complex formed by

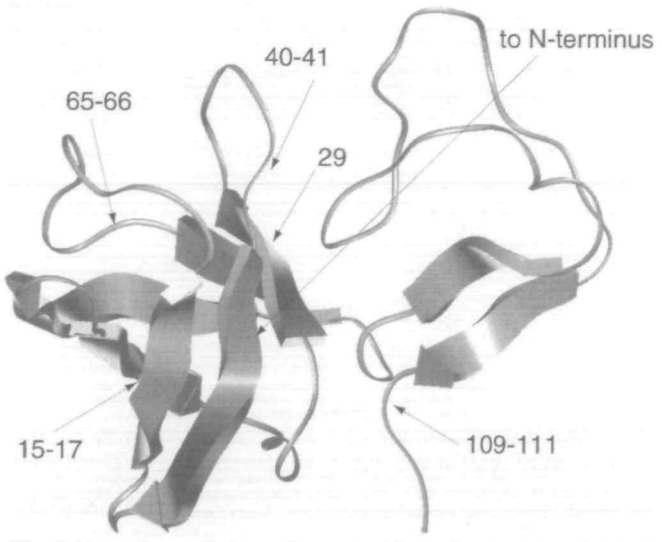

Fig. 5. **Locations of the epitope residues for β 210 and β 208 on the atomic structure of the β subunit of bovine F_1.** The amino-terminal end of bovine β (β without nucleotide) is shown as a view from the C-terminal end according to the atomic structural data (accession number of Protein Data Base: 1 bmf) (*10*). Numbers are for residues of *E. coli* β. Residue numbers of *E. coli* β correspond to those of bovine β as follows: *E. coli* Val-15 (eVal-15), bovine Val-23 (bVal-23); ePhe-17, bPhe-25; eGly-65, bGly-72; eHis-110, bHis-117.

three pairs of α and β (*11*, *13*). A dynamic conformational change was estimated to be brought about in the central catalytic domain of β (*10*, *11*) based on extensive differences in the conformations of the three βs of F_1 (*10*), which contain no nucleotides, ATP or ADP. The amino terminal β barrel structure has been thought to stabilize the expected dynamic conformational change in the central catalytic domain. However, this stabilization mechanism is not fully understand. Here, we were able to show that the surface residues of the two β strands recognized by β 210 are not essential for the function of the amino-terminal β barrel region. In this connection, it should be noted that we have shown the structural importance of residues 40 and 41 in the molecular assembly of α and β (*15*). These residues are located on a β sheet located close to the two β strands recognized by β 210 (Fig. 5), but are on the opposite side of the β barrel.

Amino acid substitutions at residues 109 and 110 cause defects in the molecular assembly of F_1, except for a His-110 to Arg mutation. This His to Arg mutation, however, causes an extensive loss of the F_1-ATPase activity. These results suggest that residues 109 and 110 play essential roles in the assembly of F_1, and that His-110 plays an important role in the catalytic mechanism as well. An Arg-111 to Ser substitution did not cause any defect in the F_1-ATPase activity, indicating that this residue is not functionally essential, consistent with no conservation of this residue among various species (Fig. 3). However, substitution of Arg-111 with Pro caused a defect in the molecular assembly, which can be explained by a drastic conformational change caused by the introduction of Pro, leading to an assembly defect in F_1. Based on the atomic structure of bovine β, the region around His-110 is close to a large loop (between residues 67 and 79) connecting the amino-terminal β barrel region and the central catalytic domain. This residue is also relatively close to the α subunit. Mutations at His-110 might cause a defect in the interaction with the loop and/or the α subunit. Since the His-110 to Arg substitution did not affect the molecular assembly, the positive charge of His can be replaced by Arg and this positive charge may play an essential role in the supposed interaction with the loop and/or α subunit. Although the functional importance of His-110 has been pointed out here, the precise mechanism involving this residue in the catalytic mechanism is not clarified at present. His-110 is obviously not involved in the catalytic center itself but might be involved in dynamic structural changes in the central domain. A subtle topological change in the positive charge of His may affect the catalytic mechanism by destabilizing the essential structure maintained by His with an as yet unidentified counter residue. Tozawa *et al.* reported (*30*) defective assembly caused by replacement of His-119 in thermophilic *Bacillus* PS3 (corresponding residue to *E. coli* His-110) with Gln and discussed the importance of this residue in stabilizing F_1 from PS3. The present study supports their suggestion by systematic analysis of the region around His-110. They showed that the C-2 proton signal of PS3 His-119 in the isolated β subunit as detected by NMR does not exhibit significant differences upon binding Mg·AMP-PNP, suggesting that this residue is not affected by the conformational change induced by nucleotide binding. However, NMR data for His-110 (PS3 His-119) in the F_1 complex is not presented in the study, and may be required in order to understand the dynamics of His-110 in the rotation mechanism.

In the present study, monoclonal antibodies β 210 and β 208 have shown to recognize surface structures of the β subunit. These antibodies will be useful for future studies of the structural and functional relationships of β in the rotation mechanism that are not fully understood in terms of the dynamic structural changes in the F_1 subunit during rotation.

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